BACKFILE DOCUMENT INDEX SHEET

A DOCPHOENIX

APPL PARTS

_____ IMIS ____
Internal Misc. Paper

_____ LET ____
Misc. Incoming Letter

_____ 371P ____
PCT Papers in a 371Application

_____ A... ____
Amendment Including Elections

_____ ABST ____
Abstract

_____ ADS ____
Application Data Sheet

_____ AF/D ____
Affidavit or Exhibit Received

_____ APPENDIX ____
Appendix

_____ ARTIFACT ____
Artifact

_____ BIB ____
Bib Data Sheet

_____ CLM ____
Claim

_____ COMPUTER ____
Computer Program Listing

_____ CRFL ____
All CRF Papers for Backfile

_____ DIST ____
Terminal Disclaimer Filed

_____ DRW ____
Drawings

_____ FOR01 ____
Foreign Reference

_____ FRPR ____
Foreign Priority Papers

_____ IDS ____
IDS Including 1449

_____ NPL01 ____
Non-Patent Literature

_____ OATH ____
Oath or Declaration

_____ PET. ____
Petition

_____ RETMAIL ____
Mail Returned by USPS

_____ SEQLIST ____
Sequence Listing

_____ SPEC ____
Specification

_____ SPEC NO ____
Specification Not in English

_____ TRNA ____
Transmittal New Application

_____ CTNF ____
Count Non-Final

_____ CTRS ____
Count Restriction

_____ EXIN ____
Examiner Interview

_____ M903 ____
DO/EO Acceptance

_____ M905 ____
DO/EO Missing Requirement

_____ NFDR ____
Formal Drawing Required

_____ NOA ____
Notice of Allowance

_____ PETDEC ____
Petition Decision

OUTGOING

_____ CTMS ____
Misc. Office Action

_____ 1449 ____
Signed 1449

_____ 892 ____
892

_____ ABN ____
Abandonment

_____ APDEC ____
Board of Appeals Decision

_____ APEA ____
Examiner Answer

_____ CTAV ____
Count Advisory Action

_____ CTEQ ____
Count Ex parte Quayle

_____ CTFR ____
Count Final Rejection

INCOMING

_____ AP.B ____
Appeal Brief

_____ C.AD ____
Change of Address

_____ N/AP ____
Notice of Appeal

_____ PA.. ____
Change in Power of Attorney

_____ REM ____
Applicant Remarks in Amendment

_____ XT/ ____
Extension of Time filed separate

Internal

_____ SRNT ____
Examiner Search Notes

_____ CLMPTO ____
PTO Prepared Complete Claim Set

_____ WCLM ____
Claim Worksheet

_____ WFEE ____
Fee Worksheet

File Wrapper

_____ FWCLM ____
File Wrapper Claim

_____ IIFW ____
File Wrapper Issue Information

_____ SRFW ____
File Wrapper Search Info

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1. Your reference — 27.68545

2. Patent application number *(The Patent Office will fill in this part)* — **9907245.6** — 29 MAR 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Andrew Goldsborough
58 Rue des Grenaches
34980 St. Gely due Fesc
France

Patents ADP number *(if you know it)* 7631591001

If the applicant is a corporate body, give country/state of incorporation

4. Title of the invention — Cleavage of Nucleic Acids from solid supports

5. Name of your agent *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
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EC4V 4EL

Patents ADP number *(if you know it)* 166001

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Hanna Dzieglewska
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68545.602

Cleavage of Nucleic Acids from Solid Supports

The present invention relates to cleavage of nucleic acids, and nucleic acid-containing molecules or constructs, from solid supports.

It is frequently desirable in many of the procedures currently in common usage in biochemistry/ biotechnology and related fields today, to bind a biological or chemical entity (e.g. a biomolecule) to a solid support (ie. to immobilise it), and then subsequently to detach it from that support. Such reversible immobilisation has notably found utility in many of the separation, purification and isolation procedures known in the art, for example isolation of cells (e.g. immunomagnetic separations), affinity chromatography etc. Immobilisation, particularly of oligonucleotides and nucleic acids, is also frequently used in many molecular biological procedures and many of the commonly-used techniques e.g. sequencing, *in vitro* amplifications, cDNA preparation, template preparation, DNA-based assays, mutagenesis procedures etc., as well as nucleic acid purification, have been adapted for use on a solid phase. This may facilitate handling, increase sample-throughput, enable automation etc., and the ability to conduct a procedure on a solid phase is frequently viewed as advantageous.

However, immobilisation may introduce its own problems. Thus, many of the immobilisation systems in use today rely on binding between a pair of affinity binding partners to achieve the linkage of the desired entity to a support. For example, this may be based on an antibody-antigen binding pair e.g. an immobilised antibody binding to a cell-surface antigen, or a protein or other molecule it is desired to immobilise. In molecular biology, binding of nucleic acids or

oligonucleotides is commonly achieved by means of a biotin-streptavidin (or avidin) linkage, the immobilised protein (streptavidin or avidin) binding to a biotinylated molecule (e.g. a biotinylated oligonucleotide).

Such linkages may be difficult to reverse (disrupt) leading to difficulties in releasing the bound moieties. Thus, for example, disruption of the linkage may require harsh conditions e.g. high pH or salt conditions, and/or high temperature, which may be harmful to the bound moieties.

Other forms of linkage/binding which may be used in place of affinity binding, e.g. linkages based on covalent bonding, may also be difficult to disrupt (e.g. binding of nucleic acids to an immobilised oligonucleotide capture probe which is covalently bound to the solid support, and hence not readily releasable from the support).

Another disadvantage of commonly used prior art immobilisation systems is that detachment of the immobilised moiety frequently results in a part of the "immobilising linkage" remaining bound to the moiety in question e.g. a binding ligand or a part thereof, for example biotin, or part of a linker arm. This may interfere in subsequent manipulation or downstream processing of the immobilised moiety, e.g. cloning or other manipulation of a nucleic acid, or viability studies on cells, conformational studies on a protein, further purification etc.

Consequently, there is a continuing need in the art for improved methods of immobilising and subsequently releasing biological or chemical entities. The present invention seeks to address this need.

A method of immobilisation has thus been developed, which is based on including in the moiety which is immobilised, a nucleotide sequence which contains a selectively cleavable site. This site is provided by a

nucleotide, not normally present in the nucleotide sequence, which is susceptible to selective cleavage, using e.g. an enzyme which is specific for the presence of that nucleotide. Detachment of the immobilised moiety may then readily be achieved by cleaving the nucleotide sequence selectively at the specified site. This novel method thus enables a nucleic acid or a molecule containing a nucleic acid, to be efficiently cleaved from the solid support at a precise site and in a predictable manner.

Since the cleavability depends upon the presence of a nucleotide, the novel method of the invention is particularly suited for use in connection with the immobilisation of nucleic acids (ie. any nucleotide sequence) and nucleic acid/nucleotide sequence manipulation and preparation procedures. However, the method may also be applied to any entity or molecule containing a nucleotide sequence into which such a cleavable site may be incorporated e.g. conjugates of other moieties such as proteins or other organic molecules with a nucleic acid.

In one aspect, the present invention thus provides a method of detaching a nucleic acid molecule from a solid support to which it is attached, wherein an unconventional nucleotide is incorporated at a pre-determined site in said nucleic acid molecule, said method comprising selectively cleaving said nucleic acid molecule at the site of said unconventional nucleotide.

The nucleic acid molecule may be any nucleotide sequence (whether a ribo- or deoxyribonucleotide sequence ie. DNA or RNA, or any modification thereof), or any molecule which contains or incorporates a nucleotide sequence. Thus not only are molecules included which consist solely of a nucleic acid(s) but also chimeric molecules which comprise a nucleic acid component and another component e.g. another molecular component such as a protein or peptide, or some other

organic molecule, a label, a ligand (ie. one of a pair of affinity binding partners), an enzyme substrate etc. e.g. any other molecular, or biological or chemical entity.

The term "nucleic acid molecule" thus includes constructs comprising a nucleic acid (nucleotide sequence) and another molecular component of a different chemical nature to a nucleic acid (e.g. a protein, lipid, carbohydrate, a small organic molecule, an inorganic molecule, a radioactive marker etc).

The term "unconventional nucleotide" means a nucleotide which does not normally occur in the nucleic acid molecule into which it is incorporated ie. in the context of a given nucleotide sequence (nucleic acid molecule) the nucleotide is not native to that sequence. Thus, for example, in the context of DNA, a nucleotide containing a uracil base (hereinafter "U") is an unconventional nucleotide, since U does not normally occur in DNA, but in RNA. Likewise, in the context of DNA, a ribose-containing nucleotide will be an unconventional nucleotide (and conversely a deoxyribonucleotide would be unconventional in RNA). Other unconventional nucleotides are modified nucleotides, which do not normally occur in nature, for example chemically derivatised nucleotides. Thus, in the context of a DNA nucleotide sequence, an unconventional nucleotide will be any nucleotide other than A, T, C or G, (but modifications of A, T, C or G are included as unconventional nucleotides) and in the context of RNA, an unconventional nucleotide will be any nucleotide other than A, U, C or G (but again including modifications thereof).

Unconventional nucleotides thus include those having "unconventional" bases, which may be modifications, derivatives or analogues of conventional bases (A, T, C, G for DNA; A, U, C, G for RNA). In addition to "unnatural" (ie. deliberately modified)

nucleotides, natural forms of nucleotides are included. Uracil and hypoxanthine, for example, are natural bases, but for the purposes of this invention, their corresponding nucleotides are unconventional nucleotides when incorporated into DNA.

"Unnatural" modified nucleotides include alkylated nucleotides and nucleotides modified by alkylhydroxylation, or any other chemical modification or derivatisation. Representative examples include N-7 methylguanine, 8-oxoguanine, deoxyuridine, deoxyinosine, deoxy 5,6-dihydroxythimine (from O_sO_4 treated DNA), 5'6'-dihydroxythine, deoxy 3'-methyladenosine and 3'-methyladenosine. Other unconventional nucleotides may include other bases observed to occur in damaged DNA, for example derivatives from ring-opened pyrimidines and other oxidative products (see e.g. Sancar and Sancar, (1988) Annu. Rev. Biochem. 57, 29-67).

An incorporated unconventional nucleotide provides a site for selective cleavage of the nucleic acid molecule in which it is incorporated. Thus the unconventional nucleotide is one which may be incorporated into a nucleotide sequence e.g. which is capable of participating in a polymerase reaction, and pairing specifically with a natural (normally occurring) nucleotide and which is capable of selective cleavage.

The terms "selective cleavage" or "selectively cleavable" etc., mean that the nucleic acid molecule may be cleaved specifically at the site of the unconventional nucleotide, and not at other sites. Thus, cleavage may be "directed", or in other words, controlled cleavage may take place at a selected site. One or more such selective sites may be incorporated in this manner, according to choice, and one or more than one unconventional nucleotide may be incorporated.

It will be understood however that, as in any biological system, absolute specificity of cleavage cannot always be guaranteed, and hence some "tolerance"

in the system is allowed for. Thus, minor or negligible instances of non-specific cleavage taking place at sites other than at the unconventional nucleotide(s) may be tolerated. What is required is that cleavage takes place substantially only at the site of the unconventional nucleotide(s).

Conveniently, such selective cleavage may be achieved enzymically, by using enzymes which are specific for the particular unconventional nucleotide in question. (The term "specific" in this context denotes the relationship between the enzymes and their corresponding substrates, and conversely, the substrates that are recognised by a corresponding enzyme). What is required is that the enzyme recognises, and cleaves at the unconventional nucleotide in an discriminating manner, and not other nucleotides (ie. conventional nucleotides present in the nucleic acid molecule).

Such specific enzymes may typically be DNA glycosylases which have specificity for a particular base or particular bases. Thus glycosylase enzymes exist which recognise and act at only one or a particular group of bases. Such enzymes are believed to be involved in base-excision DNA repair processes, and may recognise and discriminate between normal and inappropriate (ie. inappropriate base pairing), or defective (damaged) bases. DNA glycosylases work by cleaving the particular base(s) recognised from the deoxyribose-phosphate backbone of the DNA by breaking the N-glycosidic bond. The abasic (apurinic/apyrimidic) site generated by this reaction is labile, and can readily be cleaved, principally by high pH or high temperature. However, a range of cleavage methods for abasic sites are available, including those using chemical reagents or enzymes to break the phosphodiester bond of the nucleotide sequence. These are discussed further below. This provides a mechanism for selective cleavage according to the present invention.

Many such enzymes have been studied and are described in the literature (see e.g. Cleaver and Layher, (1995) Cell, 80, 825-827; and Sancar and Sancar, supra). Any such enzyme may be used in the present invention, together with its corresponding substrate as the unconventional nucleotide.

However, the invention is not limited to the use of enzymes to achieve selective cleavage, and any suitable mechanism or system for cleavage may be used. Thus, for example, a ribo-nucleotide incorporated into a DNA nucleotide sequence is sensitive to conditions such as high pH and ions such as Mg^{2+} and Mn^{2+} and may provide a site for selective cleavage achieved simply by such treatment. Sequences consisting solely of deoxyribonucleotides (e.g. dT, C, G, A, U) are not affected by such treatment. The presence of ribose instead of deoxyribose therefore offers a method to cleave selectively at one position. Example 3 below describes the utility of such a cleavage system in a method of cDNA synthesis.

In preferred embodiments of the invention, the nucleic acid molecule is a DNA molecule, the unconventional nucleotide is U and the selective cleavage is achieved using a Uracil DNA glycosylase (UDG) enzyme (also known as uracil N-glycosylase or UNG). This may be a natural enzyme or a modified enzyme and a variety of UDG enzymes, both natural and modified, are known in the art and described in the literature (see e.g. Cleaver and Layher, supra, and US-A-5,035,996; US-A-5,536,649 and Longo et al., Gene (1990), 93, 125-128, which describe the use of UDG in controlling carry-over contamination in PCR and provide sources for UDG enzymes; see also WO92/01814, which describes PCR carry-over control using other glycosylase enzymes, as well as UDG, and unconventional nucleotides and also describes a range of different UDG enzymes, and their sources which may be used in the present invention). Both

thermostable (Koulis, A. et al., FEMS Microbiology Letters 91996) 143, 267-271; Kaboev, O.K. et al., Letters (1981) 132(2), 337-340) and thermolabile forms (HK™ UNG, EpiCentre Technologies Inc.) are known. UDG is available from commercial sources such as Amersham Life Sciences, UK, Boehringer Mannheim, Germany and EpiCentre Technologies Inc., USA.

Glycosylase genes have been identified and cloned. (J. Biol. Chem., 1984, (259): 13723-13729 [*E. coli*], and J. Biol. Chem., 1989, 264: 9911-9914 [human]). In place of UDG, other glycosylase are available which could be used. These include: hypoxanthine-DNA glycosylase, 3-methyladenine-DNA glycosylase I, 3-methyladenine-DNA glycosylase II, hydroxymethyl uracil-DNA glycosylase and formamido-pyrimidine DNA glycosylase. Illustrative glycosylases and corresponding substrates are shown in the table below.

DNA Glycosylases

Enzyme	DNA Substrate Containing
Uracil-DNA glycosylase	Uracil
Hx DNA glycosylase	Hypoxanthine (Hx)
3-mA DNA glycosylase I	3-methyladenine (3-mA)
3-mA DNA glycosylase II	3-methyladenine, 7-methyl-guanine or 3-methylguanine
FaPy DNA glycosylase	Formamido-pyrimidine (FaPy) moieties
5,6-HT DNA glycosylase	5,6-hydrated thymine (5,6-HT) moieties
Urea DNA glycosylase	Urea moieties
PD DNA glycosylase	Pyrimidine dimers (PD)
5-HmU DNA glycosylase	5-hydroxymethyl uracil (5-HmU)
5-HmC DNA glycosylase	5-hydroxymethyl cytosine (5-HmC)

The unconventional nucleotide is incorporated and cleaved at a pre-determined site. This means simply that the site of incorporation (and hence cleavage) may be pre-selected (or controlled or directed) such that cleavage can take place at a precise site and desired location in the nucleic acid molecule. The unconventional nucleotide may be incorporated at a defined position(s) of the nucleic acid molecule.

As mentioned above, the method of the invention has utility in any method of immobilisation involving a nucleic acid molecule.

In another aspect, the invention accordingly provides a method of reversibly immobilising a nucleic acid molecule, said method comprising:

(a) incorporating an unconventional nucleotide into said nucleic acid molecule at a pre-determined site;

(b) binding said nucleic acid molecule to a solid support; and subsequently

(c) selectively cleaving said nucleic acid molecule at the site of said unconventional nucleotide.

The selective cleavage step (c) results in detachment of the nucleic acid molecule from the support.

Conveniently, in the methods of the invention, the unconventional nucleotide(s) may be introduced as part of a polynucleotide or oligonucleotide sequence which is introduced into or incorporated into the nucleic acid molecule.

This nucleotide sequence may be viewed as a "linker sequence" which is composed of nucleotides and incorporates the unconventional nucleotide at one or more positions, which may be contiguous or non-contiguous. This "linker sequence" may be e.g. 4 to 100 nucleotides in length, for example 5 to 50 and conveniently 10-30 or 12-21, and may be composed entirely of the unconventional nucleotide, or may

comprise one or more unconventional nucleotides at different positions, according to choice.

Procedures for synthesising poly- and oligonucleotide sequences for use in this regard are well known in the art and described in the literature, and any known method may be used. The unconventional nucleotide may be incorporated into the linker in any convenient way, for example by solid phase synthesis using appropriate nucleotides, or enzymically, e.g. by incorporation of approximately provided nucleotides using a polymerase enzyme. Alternatively, the unconventional nucleotide may be incorporated into the linker using a specific enzyme e.g. a transferase enzyme, such as terminal deoxynucleotidyl transferase which can add a mononucleotide such as UMP from the corresponding NTP (in this case UTP) precursor.

Likewise, the linker sequence may be introduced into the nucleic acid molecule in any convenient or desired way, known and described in the literature, for example by ligation, or in the form of a primer for a template-directed polymerase-catalysed chain extension reaction, wherein the primer (ie. the linker) becomes incorporated into the chain extension reaction product. In other words, the nucleic acid molecule of the invention, may be a primer-extension product. This is discussed further below.

Processes for coupling nucleic acids to other molecules are also known in the art, and are discussed in more detail below.

The solid support may be any of the well-known supports or matrices which are currently widely used or proposed for immobilisation, separation etc. These may take the form of particles, sheets, gels, filters, membranes, or microtitre strips, tubes or plates, fibres or capillaries, and conveniently may be made of a polymeric material e.g. agarose, cellulose, alginate, teflon, latex or polystyrene. Biochips may be used as

solid supports to provide miniature experimental systems as described for example in Nilsson et al. (Anal. Biochem. (1995), 224, 400-408). Particulate materials, especially beads, are generally preferred, particularly polymeric beads, a wide range of which are known in the art. To aid manipulation and separation, magnetisable ("magnetic") beads are preferred. Preferably such magnetic particles are superparamagnetic to avoid magnetic remanence and hence clumping, and advantageously are monodisperse to provide uniform kinetics and separation. The preparation of superparamagnetic monodisperse particles is described by Sintef in EP-A-106873.

The monodisperse polymeric superparamagnetic beads sold as DYNABEADS by Dynal AS (Oslo, Norway) are particularly suitable for use according to the invention.

The nucleic acid molecule may be bound, or attached, to the solid support by any convenient means, e.g. any physical or chemical means, as long as the unconventional nucleotide(s) is (are) accessible to cleavage. This may include, for example, by electrostatic e.g. hydrogen bonding, entrapment, or more conveniently by covalent bonding, which may be achieved for example by covalent bonding between a group on the support surface and a "linker" or functional bonding group at the 5' end of the nucleic acid molecule. The nucleic acid molecule may also be bonded directly or indirectly to other molecules which may themselves be bound to the solid insoluble support by any of the above-mentioned means.

Methods for binding nucleic acids to solid support are described in the literature, as are methods for modifying or adapting the surface of solid supports to carry, or bind nucleotide sequences. (For a general review, see for example Bioconjugate Techniques by Greg T. Hermanson, Academic Press, Inc. 1996 ISBN 0-12-34

2335, Chapter 17, Nucleic Acid and Oligonucleotide Modification and Conjugation, and Bioconjugation - Protein Coupling Techniques for the Biomedical Sciences by Mohammed Aslam and Alastair Dent, Macmillan Reference Ltd., 1998 ISBN 0-333-583752, Chapter 7, The Preparation of Protein-Nucleic Acid Conjugates).

The binding of oligonucleotides to magnetic beads is described, for example, in EP-A-0640626.

In addition to direct, covalent, coupling, the nucleic acid may be bound to the solid support indirectly by means of an affinity binding pair, one binding partner being on the solid support, and the other on the nucleic acid molecule to be immobilised. The use of the biotin-streptavidin (avidin) system is well known in molecular biology as a means of immobilising nucleic acids and biotinylated nucleotides are readily available, as are solid supports carrying streptavidin. For example, streptavidin-coated magnetic beads are available from Dynal AS, Oslo, Norway and streptavidin-coated tubes and microwell plates are available from Roche Molecule Biochemicals. Different affinity binding partners may be used in place of biotin/streptavidin, for example an antigen/hapten and antibody, enzyme and substrate etc. A range of commercially available products exist in this regard also, for example anti-digoxigenin antibody carrying magnetic particles, anti-fluorescein antibodies, and anti-biotin antibodies from Roche Molecular Biochemicals. The nucleic acid molecule may also incorporate a nucleotide sequence (a target site) which is recognised by a specific DNA binding probe carried on the support, e.g. the lac repressor LacI, binding to the lac operon.

Incorporating the unconventional nucleotide by way of a primer represents a preferred embodiment according to the invention. As mentioned above, this provides a ready way of incorporating an unconventional nucleotide

and preparing the nucleic acid molecule which is to be immobilised; the nucleic acid molecule is formed by extending the primer in a chain-extension reaction, and the unconventional nucleotides are provided, incorporated in the primer. This simply involves including the unconventional nucleotide (NTP) in the primer sequence e.g. replacing a conventional nucleotide with an unconventional one at one or more defined positions in a synthetic DNA primer. Such a procedure forms the basis for a number of uses of the present invention.

The primer extension reaction may be any such reaction known in the art e.g. a simple linear extension reaction, be this DNA or RNA synthesis. Thus not only is DNA synthesis from a DNA template covered, but also DNA synthesis from an RNA template (reverse transcription), RNA synthesis from a DNA template etc. Also included are *in vitro* amplification reactions based on a primer-extension principle, whether exponential or not, e.g. PCR and modifications thereof etc. Primer extension reactions form the basis of a number of molecular biological procedures in usage today. Thus, the nucleic acid molecule may be a PCR product, a sequencing product, a cDNA synthesis product or a template-generation product etc.

For convenience, a linker sequence (e.g. a primer) may be used which is provided with means for immobilisation to a solid support. Such means may be an affinity binding partner e.g. biotin, capable of binding to the corresponding binding partner, which may be provided on the solid support, or a functional group capable of undergoing a chemical coupling reaction e.g. an amino group or a terminal nucleoside which may interact with a carboxyl group or a CNBr-activated hydroxyl group on the solid support.

Biotinylated poly- or oligonucleotides incorporating an unconventional nucleotide form a

particularly preferred aspect according to the invention, but this aspect of the invention also includes poly- or oligonucleotides incorporating an unconventional nucleotide, provided with any means for immobilisation.

In the PCR (or similar) embodiments of the invention, the PCR reaction may be carried out before or after immobilising the primers on the solid support; the PCR primers may be used already bound to the solid support, rather than simply provided with means for immobilisation.

Thus, also included in the scope of the invention are immobilised poly- or oligonucleotides incorporating an unconventional nucleotide. Preferably, such immobilised poly- or oligonucleotides are bound to a magnetic bead, preferably via end-attachment, and preferably they are 5'-end attached.

By way of example, a representative PCR reaction may be described which takes advantage of the present invention.

Biotinylated, synthetic oligonucleotides with dUMP replacing dTMP at one or several positions (e.g. biotin-GTAATACGACTCACTAUAGGGC) can be used for PCR and incorporated into a dsDNA product in a manner identical to dTMP containing oligonucleotides. Following PCR and binding to a solid support the internal dUMP site is a target for UDG activity. The abasic site can then be cleaved by high temperature or high pH, releasing the PCR product from the solid support. Alternatively, the non-biotinylated, complementary strand can first be released by heat denaturation, leaving the biotinylated strand immobilised to the solid support. The single stranded DNA can then be cleaved with UDG, releasing it, and allowing both strands to be separately collected and purified. This could be useful when sense and anti-sense strands are required for hybridisation probes or cDNA subtraction library preparation.

Analogous procedures may be performed using other unconventional nucleotides/cleavage systems, and/or other primer extension reaction e.g. a sequencing primer-based reaction.

Another important use of the present invention is in the preparation of cDNA. It is now established in the art to isolate mRNA for cDNA synthesis by using an oligonucleotide capture probe capable of binding to the desired mRNA. The bound capture probe may then be used as a primer in a subsequent cDNA synthesis reaction using reverse transcriptase (see e.g. EP-A-0444119 of Dynal AS) which describes such a cDNA synthesis approach based on a solid phase. Such a capture probe/primer may constitute the "linker sequence" of the present invention.

For isolating total mRNA, an oligo dT capture probe/primer may conventionally be used to bind to the poly A tail present on all mRNAs. Such an oligo dT may be adapted for use according to the present by incorporating one or more unconventional nucleotides e.g. U. Advantageously, a poly- or oligo dU may be used. This has the advantage of being simple to manufacture.

It should be noted that oligo (dU) base pairing is stronger than oligo (dT) for the poly (A) tail of the mRNA, offering a distinct advantage for its use because it should improve mRNA capture.

Accordingly, further preferred aspects of the invention provides an oligo- or poly dU provided with means for immobilisation to a solid support, preferably biotin, or bound to a solid support, preferably magnetic beads.

Removal of cDNA products from a solid support thus represents a preferred embodiment of the invention.

mRNA templates, immobilised on a solid support carrying an oligo (dT) can be copied into cDNA. During this reverse transcription reaction, the cDNA produced

becomes covalently bound to the solid support via the oligo (dT) primer. Release of the cDNA is therefore difficult using current methods. By incorporating dUMP into the oligo (dT) (e.g. 5'TTTTTTTTTTTTTTTTTTTTTU), a site is produced that is a target for UDG activity. Subsequent cleavage of the abasic site by high temperature or pH releases the cDNA from the solid support. Alternatively, multiple target sites can be introduced (e.g. 5' UUUUUUUUUUUUUUUUUUUUU). Following reverse transcription, it may be convenient or advantageous to remove the RNA strand e.g. by RNase digestion such as RNase H or by any other means, such as high pH, prior to releasing the bound cDNA product by UDG cleavage.

In addition to primer-extension-based methods, the methods of the invention may also be in connection with other *in vitro* amplification methods, such as the ligase chain reaction (LCR) and the Qβ Replicase system (WO 87/06240). Thus the nucleic acid molecule may also be formed by other means e.g. ligation. The unconventional nucleotide(s) may be included in one or more of the oligonucleotides which are ligated in the LCR reaction; the LCR oligonucleotides may thus serve as the linker sequences according to the invention.

In the above-described embodiments of the invention the nucleic acid molecule may be, principally, a DNA molecule, or a DNA-RNA molecule. However, it will be understood that other forms of construct are also possible, for example a "linker sequence" provided with means for immobilisation at one end, and linked at the other end to, for example, a further DNA sequence, an RNA sequence, an RNA-DNA sequence or double-stranded DNA. Thus, various forms of a solely "nucleic acid-containing" nucleic acid molecule are possible. Such nucleic acid molecules may be prepared in any conventional or convenient manner, according to techniques well known in the art. For example, RNA-DNA

constructs may readily be synthesised using an oligonucleotide synthesizer. Alternatively, RNA and DNA may be joined using a RNA ligase (which will also join DNA to DNA and RNA to RNA). Individual dNTP's or rNTP's may be enzymatically introduced into a nucleotide chair using a suitable polymerase enzyme e.g. a mutant DNA polymerase such as T7 DNA polymerase (Sausa and Padilla (1995) EMBO J. 14, 4609-4621).

In addition, as mentioned above, other constructs, or chimeric forms of nucleic acid molecules are possible.

Thus, a nucleic acid molecule may conveniently comprise a linker sequence coupled to a protein (or a peptide or polypeptide), which might, for example, be an antibody or a fragment thereof (including antibody derivatives and synthetic antibodies such as single chain antibodies), an enzyme or a receptor protein or some other binding protein or binding portion or fragment thereof (e.g. streptavidin, protein A, protein G, protein L, or fragments thereof or indeed any known, synthetic or modified (e.g. genetically modified) affinity binding protein such as antibodies, lectins etc.). Alternatively, the linker sequence may be coupled to an enzyme substrate, a receptor ligand, an antigen/hapten or fragment thereof etc. Advantageously, therefore, the "second" component of the chimeric nucleic acid molecule is an affinity binding group i.e. one of a pair of affinity binding partners.

Such chimeric nucleic acid molecules have utility in any solid phase process or procedure based on affinity binding, for example in separation and purification procedures, e.g. of cells or proteins or other molecules, or in assays.

A further aspect of the invention thus provides a method of preparing a construct for binding to, and subsequent cleavage from, a solid support, said method comprising incorporating into said construct a

nucleotide sequence comprising at a pre-determined site an unconventional nucleotide capable of selective cleavage.

In a still further aspect, the present invention also provides a chimeric molecule (or construct) comprising a nucleotide linker sequence comprising a selectively cleavable unconventional nucleotide at a pre-determined site, coupled to a functional group, preferably an affinity binding group or a reporter group.

Advantageously, in such a chimeric molecule the linker sequence is further either immobilised (i.e. bound to a solid support) or provided with means for immobilisation to a solid support, as discussed above.

The functional group may be any group having a property e.g. a binding activity or a detectable, or signal-giving activity useful in an assay or separation procedure. Advantageously, as mentioned above, the functional group is an affinity binding group. Alternatively, it may be a reporter group. This is defined herein to include any group capable of providing a detectable signal, or participating in a signal-generating reaction. Thus a group might for example include an enzyme substrate, or a marker or label, e.g. a radioactive label.

The invention also includes constructs in which the affinity binding group is itself further provided with a reporter group e.g. a label. This may be any label known in the art or described in the literature, e.g. a radioactive label or some other detectable label e.g. a coloured, pigmented, chromogenic, fluorescent or chemiluminescent label, or an enzyme marker.

Representative functional groups will include an antibody or a binding protein such as streptavidin which may optionally be labelled; an enzyme, e.g. horseradish peroxidase (HRP) or alkaline phosphatase (AP); an enzyme substrate, e.g. a peptide, which may be a

substrate for an enzyme under investigation e.g. HIV proteinase or a tyrosine kinase phosphorylation site; or indeed any substrate (e.g. testosterone or some other biomolecule) for an enzyme assay; a carbohydrate; a lipid; a hapten (e.g. a small organic molcule); and a label.

In such constructs the linker sequence is preferably an oligo- or poly- dU sequence, preferably carrying a biotin group as means for immobilisation.

A preferred functional group is an antibody or a fragment thereof, or a hapten.

Methods for coupling nucleotide sequences to other molecules are well known in the art, and such constructs may be prepared following, for example, the methods described in Hermanson (supra) and Aslam and Dent (supra). General guidance to procedures and methods for coupling oligonucleotides (which may be modified) to other molecules may also be found in R. Helmuth (1990) PCR Protocols: A Guide to Methods and Application, Chapter 15, Academic Press, Inc. and Y.M. Dennis Lo. et al., (1990) PCR Protocols: A Guide to Methods and Application, Chapter 14, Academic Press, Inc..

It is well known that "modified" oligonucleotides may be prepared by attaching additional chemical groups to the oligonucleotides during synthesis, for example a reporter group, e.g. a label, such as a fluorescent label e.g. TAMRA, rhodamine or any other known fluorescent label. Such a chemical group may also be an affinity binding partner as discussed above, e.g. a hapten such as digoxigenin, estradiol or dinitrophenol (DNP) providing a target for binding with antibodies, or biotin (binding with streptavidin/avidin) or an entity which may be both a reporter group and an antigen/hapten for binding with antibodies e.g. fluorescein. Many such modified oligonucleotides are available commercially e.g. from Oswell Research Products Ltd., Southampton UK. As explained above, in the chimeric molcules of the

invention a single oligonucleotide may carry more than one additional "chemical group" (or "modified unit") e.g. an immobilising moiety (ie. means for immobilisation) such as biotin and an affinity binding group (e.g. a hapten) or a reporter group (e.g. a label). For example, a chimeric molecule of the invention may take the form of an oligonucleotide carrying a biotin molecule at the 5' end and an amino group at the 3' end - the amino group may be used for e.g. labelling, as it provides a means to attach amino-reactive molecules to the oligonucleotide. Alternatively, at its 3' end the oligonucleotide may carry a hapten such as digoxigenin. For labelling, the digoxygenin may be coupled to an enzyme such as horse radish peroxidase (HRP) via an anti-digoxygenin antibody.

The constructs may be used in separation procedures using standard well-known techniques. Thus, for example, in the case of an antibody functional group, the construct may be used to bind to any target entity e.g. a cell or a protein, which it is desired to separate. Immobilisation of the construct, either before or after binding to the target entity, facilitates separation of the target entity, following which the target entity may be released by cleaving the linker sequence, according to the present invention.

For example, in a protein purification procedure, an affinity binding group (ie. ligand) for the protein in question may be coupled to a solid support via an oligonucleotide "linker" sequence according to the invention. In this construct, the chimeric molecule of the invention thus comprises, means for immobilisation, a cleavable "linker sequence", and an affinity capture ligand for the desired target protein.

Exemplificatory affinity capture ligands include L-thyroxin in order to purify thyroxine-binding protein (Pensky and Marshall (1969) Arch. Biochem. Biophys.

135:304), folic acid to capture folate-binding protein (Salter et al., (1972) FEBS Lett. 20:302) and cholesteryl hemisuccinate to capture lipoproteins (HDL and LDL) (Wichman (1979) Biochem. J. 181:691). In each case the ligand would be cleaved from the support allowing the collection of the protein and attached ligand. The ligands could also be small molecules such as L-lysine, proteins as in protein A, carbohydrates such as D(+) melibiose or lectins such as concanavalin A. Nucleotide ligands are particularly favoured for enzyme purification because one-third of all enzymes require a nucleotide coenzyme thus providing a means for purification (Barker et al., (1972) J. Biol. Chem. 247: 7235). Ligand-protein interaction may be enhanced by use of a spacer arm between the cleavable oligonucleotide linker sequence and the ligand in order to reduce steric hinderance. For example, nucleotide ligands are frequently immobilised by attachment via the base such as N-6 for adenosine 5' monophosphate with a spacer arm of 8, 9 or 11 atoms (Catalogue No. A3019, Sigma-Aldrich Companh) (Chaffotte et al., (1977) Eur. J. Biochem. 78:309).

Similar or analogous principles may be used in assays, for example ELISA assays, or another of the assay principles known in the art.

The ability afforded by the present invention to cleave an antibody-enzyme complex from a solid support finds utility in ELISA assays. Normally in an assay such as an ELISA, only one ligand (ie. assay target) can be detected and quantitated per well. However modifications may allow several ligands to be detected by a multiplexing approach. For example, two or more ligands (in this case the assay targets) may be captured in the well of the ELISA plate at the sme time using antibodies specific for each ligand. A secondary antibody specific for the first ligand may then be added which is linked to an enzyme or other method of

detection via a cleavable linker sequence according to the present invention, such as poly(dU). Following processing of the well as normal, the enzyme may be separated from the complex and washed away by making use of the cleavable linker. This allows the second secondary antibody specific for the second ligand to be added to the well, and an enzyme assay conducted as for the first. The whole process may be repeated several times allowing multiplexing of the ELISA assay.

The use of the reversible immobilisation method of the invention in a cell isolation procedure represents a preferred and advantageous aspect.

Accordingly, this aspect of the invention provides a method for separating a target cell from a sample, said method comprising binding said target cell to a solid support by means of a chimeric molecule as hereinbefore defined wherein said functional group is an affinity binding group which binds specifically to said cell. Cells separated in this manner may be released from the solid support by cleaving said nucleotide linker sequence.

More particularly this aspect of the invention therefore comprises:

(a) binding said target cell to a solid support by means of an affinity binding partner which binds specifically to said cell, the linkage between said binding partner and said support comprising a nucleotide sequence comprising a selectively cleavable unconventional nucleotide at a pre-determined site;

(b) separating the support-bound cell from the sample; and

(c) releasing said target cell from the support by selectively cleaving said nucleotide sequence at the site of said unconventional nucleotide.

The term "cell" is used herein to include all prokaryotic (including archaebacteria) and eukaryotic cells and other viable or membrane-enveloped entities

such as viruses and mycoplasmas, and sub-cellular components such as organelles. Representative "cells" thus include all types of mammalian and non-mammalian animal cells, plant cells, protoplasts, bacteria, protozoa and viruses.

The sample may thus be any material containing such cells, including for example foods and allied products, clinical and environmental samples. Thus, the sample may be a biological sample, which may contain any viral or cellular material, including all prokaryotic or eukaryotic cells, viruses, bacteriophages, mycoplasmas, protoplasts and organelles. Such biological material may thus comprise all types of mammalian and non-mammalian animal cells, plant cells, algae including blue-green algae, fungi, bacteria, protozoa etc. Representative samples thus include whole blood and blood-derived products such as plasma or buffy coat, urine, faeces, cerebrospinal fluid or any other body fluids, tissues, cell cultures, cell suspensions etc., and also environmental samples such as soil, water, or food samples.

The sample may also include relatively pure or partially purified starting materials, such as semi-pure preparations obtained by other cell separation processes.

In the case where enzymic cleavage using a DNA glycosylase is used to release the bound cells, it may in some circumstances be desirable to avoid the more aggressive cleavage conditions (e.g. high temperature and/or pH) which are conventionally used to cleave the abasic site created by glycosylase action. Thus, in order to achieve cleavage under more physiological conditions, favourable to living cells, DNA glycosylases may be used in a cell detachment protocol based on a combination of enzymatic cleavage under physiological conditions with mechanical stress to break the enzyme-digested (ie. degraded) nucleotide linker. Such

mechanical stress may be provided externally e.g. by stirring, mixing, pipetting or agitating etc., but simply the mechanical stress expected on the linkage by the linked entities, ie. by the cell and the solid support, may suffice.

However, if desired, further methods may also be used to enhance cleavage of the abasic site generated by DNA glycosylase action, including the use of "cleavage agents" such as chemical reagents or enzymes, for example exonuclease III or *E. coli* endonuclease IV, which cleave at abasic sites. This also enables mild cleavage procedures to be adopted. Such cleavage methods are exemplified further in the Examples below.

The components, or means, required to perform the method of the invention may conveniently be provided in the form of a kit.

Accordingly, a further aspect of the invention provides a kit for use in the methods of the invention as hereinbefore defined, said kit comprising

(a) means for introducing an unconventional nucleotide into a nucleic acid molecule; and

(b) means for selective cleavage of said unconventional nucleotide.

Means (a) may conveniently be an oligonucleotide sequence containing said unconventional nucleotide e.g. an linker sequence as described above, preferably a primer.

Means (b) may conveniently be a DNA glycosylase enzyme.

The kit may additionally comprise further components such as a solid support, or means for immobilisation to a solid support. Preferably, the kit may comprise an oligo- or poly dU provided with means for immobilisation to a solid support, preferably biotin, or bound to a solid support, preferably magnetic beads.

Uses and applications of the invention include the

following:

1. First and second strand cDNA can be made on the solid support and then released by the selective cleavage, for cloning purposes such as library preparation, or for probe preparation for use in hybridisation protocols e.g. with biochips (gene chips), and to remove unincorporated dNTP's following sequencing reactions, but prior to loading.

2. Both sense and anti-sense strand probes can be prepared from the same ds cDNA preparation, simplifying the process.

3. Reverse subtraction hybridisation is possible, the first strand cDNA can be removed from the support and hybridised with immobilised mRNA on a solid support.

4. DNA such as PCR products can be cloned, a major advantage because normally there is a biotin molecule at the 5' end which would inhibit ligation.

The invention will now be described further in more detail in the following non-limiting Examples with reference to the drawings in which

Figure 1 is a graph showing % DNA released with dTMP or dUMP oligonucleotide primers treated with UDG; and

Figure 2 is a graph showing release of dUMP PCR primer-product (CPM released (000's) in the presence or absence of UDG.

Example 1

Removal of cDNA products from a solid support.

Methods

1. Capture, wash and prepare 1μg mRNA using Dynabeads Oligo 5'TTTTTTTTTTTTTTTTTTTU or 5'UUUUUUUUUUUUUUUUUUUU as described (Jakobsen, K.S. et al. In Advances in Biomagnetic Separation, Ed. Uhlén, M. Eaton Publishing, (1994) pp61-71) or according to Dynal's instructions for mRNA capture.

2. Wash mRNA and bead complex twice in 50μl 1X reverse

transcription buffer (Life Technologies, Inc.).

3. Mix the 20μl reverse transcription reaction containing 200 units of Super Script II reverse transcriptase in 1 X reverse transcriptase buffer containing 500nM dNTP's, ^{33}p dATP (3000mCi/mmol.), 100 mM DTT and 2.5 mM $MgCl_2$ (Life Technologies, Inc.).

4. Incubate at 37°C for 20 min., 42°C for 20 min. and 55°C for 20 min.

5. Incubate at 37°C for 15 min. with 1μl of RNaseH and 70°C for 10 min.

6. Wash twice with 50μl UDG reaction buffer (10mM Tris-HCl, pH 8.9, 50mM KCl and 25mM $MgCl_2$).

7. Add 1 unit Uracil-DNA glycosylase (Boehringer mannheim) in 20 μl UDG reaction buffer and incubate 10 minutes at 37°C.

8. Incubate at 95°C for 3 minutes to cleave DNA at abasic sites.

9. Apply magnet, collect supernatant containing released cDNA.

RESULTS

Figure 1 demonstrates the marked increase of labelled first strand cDNA released when UDG is incubated with oligo (dTU) compared with oligo (dT). Approximately 80% of the cDNA is released compared with less than 20% respectively.

Example 2

Removal of PCR products from M-280 Streptavidin beads.

Methods

1. Carry out PCR containing one biotinylated primer with an internal dUMP such as biotin-GTAATACGACTCACTAUAGGGC and an unmodified PCR primer with a suitable template and reaction components (no changes need to be made when using a biotinylated and dUMP containing PCR primer compared with the same unmodified primer).

2. Unincorporated biotinylated primers can be removed with a Centricon-50 spin column (Amicon Inc.) using manufacturer's instructions.

3. Immobilise the purified PCR product (100ng-5μg) on 100μl M-280 Streptavidin magnetic beads (Dynal) in 100 μl B & W buffer (2M NaCl, 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA) at 50°C for 3 hrs with rotation.

4. Proceed with steps 6-9 from Example 1 above.

5. The released double or single stranded stranded PCR product can be collected and utilised for downstream applications such as cloning.

RESULTS

Figure 2 shows release of radiolabelled biotinylated PCR products from M-280 streptavidin beads.
bioT3U is biotin-AATTAACCCTCACUAAAGGG
bioT7U is biotin-GTAATACGACTCACTAUAGGGC
A greatly increased amount of PCR product is released in the presence of UDG than without.

Example 3

Removal of cDNA products from a solid support using a ribonucleotide as an unconventional nucleotide

1. Capture, wash and prepare 1 μg mRNA using Dynabeads Oligo 5'biotin TTTTTTTTTrUTTTTTTTTT as described (Jakobsen et al. 1994).

2-4. as for Example 1.

5. Cleave the RNA (rU) containing oligonucleotide and release the attached cDNA by the addition of two lots of 20 μl 100 mM NaOH.

6. Apply magnet, collect supernatant containing released cDNA.

7. Neutralise the alkali if necessary by the addition of 40 μl of 100 mM HCl or a suitable volume of a buffered 1M solution (pH 7) of Tris-HCl.

Example 4

Removal of cDNA products from a solid support using a ribonucleotide as an unconventional nucleotide

1-4. As for Example 3 above.

5. Cleave the RNA (rU) containing oligonucleotide and release the attached cDNA by the addition of 10 μl of 25 mM $MgCl_2$ or $MnCl_2$ solution to bring the final Mg^{2+} or Mn^{2+} concentration to at least 10 mM. Heat at 94°C for 5 miunutes.

6. Apply magnet, collect supernatant containing released cDNA.

Example 5

Use of exonuclease III to cleave apurinic sites

Methods

Steps 1-7 as described for Example 1.

8. Add 150 units of Exonuclease III (Promega Corp. USA) and incubate for 30 minutes at 37°C.

9. Apply magnet, collect supernatant containing released cDNA.

Notes. Exonuclease III belongs to a class of abasic site cleaving enzymes collectively known as AP nucleases (Lindahl and Anderson (1972) Biochemistry 11:3618-3623). Exonuclease III also contains a double strand specific DNase activity that will destroy double stranded DNA such as PCR products, but not ssDNA. Therefore it is preferable to cleave abasic sites with exonuclease III only in single stranded DNA as described in this Example. Another suitable enzyme for cleaving abasic sites is *E. coli* Endonuclease IV (Demple and Harrison, (1994) Annu. Rev. Biochem. 63:915-948) which may be substituted for Exonuclease III in this Example.

Example 6

Linker Cleavage and Cell Purification

1. Perform cell isolation with biotin-polyU-anti-CD4 as described for Dynabeads CD4 (Cell separation and protein purification, Technical Handbook, 2nd edition, Dynal).
2. Resuspend the rosetted cells in 100 ml of cell culture medium.
3. Add 100 units of UDG (Roche Molecular Biochemicals) and 1500 units of exonuclease III.
4. Incubate for 45-60 minutes at 37°C.
5. Remove the released beads using a magnetic separator.
6. Collect the supernatant containing the cells.

Example 7

Linker Cleavage and Protein Purification

Method

1. Wash 1 x 107 tissue culture cells 3 times in PBS.
2. Lyse cells in 1 ml 2% Triton X-100 containing 50 µg/ml TLCK and TPCK and 200 µg/ml PMSF.
3. Incubate cell lysate with 40 µl of Dynabeads-cleavable linker-affinity molecule (e.g. Dynabeads-poly(dU)-8-carbon spacer-adenosine 5' monophosphate).
4. Collect beads using magnet and wash 3 x with PBS.
5. Resuspend beads in 40 µl of UDG reaction buffer (10 mM Tris-HCl, pH 8.9, 50 mM KCl and 25 mM MgCl2.
6. Add 2 units Uracil-DNA glycosylase (Boehringer Mannheim).
7. Incubate 30 minutes at 37°C.
8. Add 15 units of Exonuclease III (Promega Corp. USA) and incubate for 30 minutes at 37°C.
9. Apply magnet, collect supernatant containing released protein and ligand.

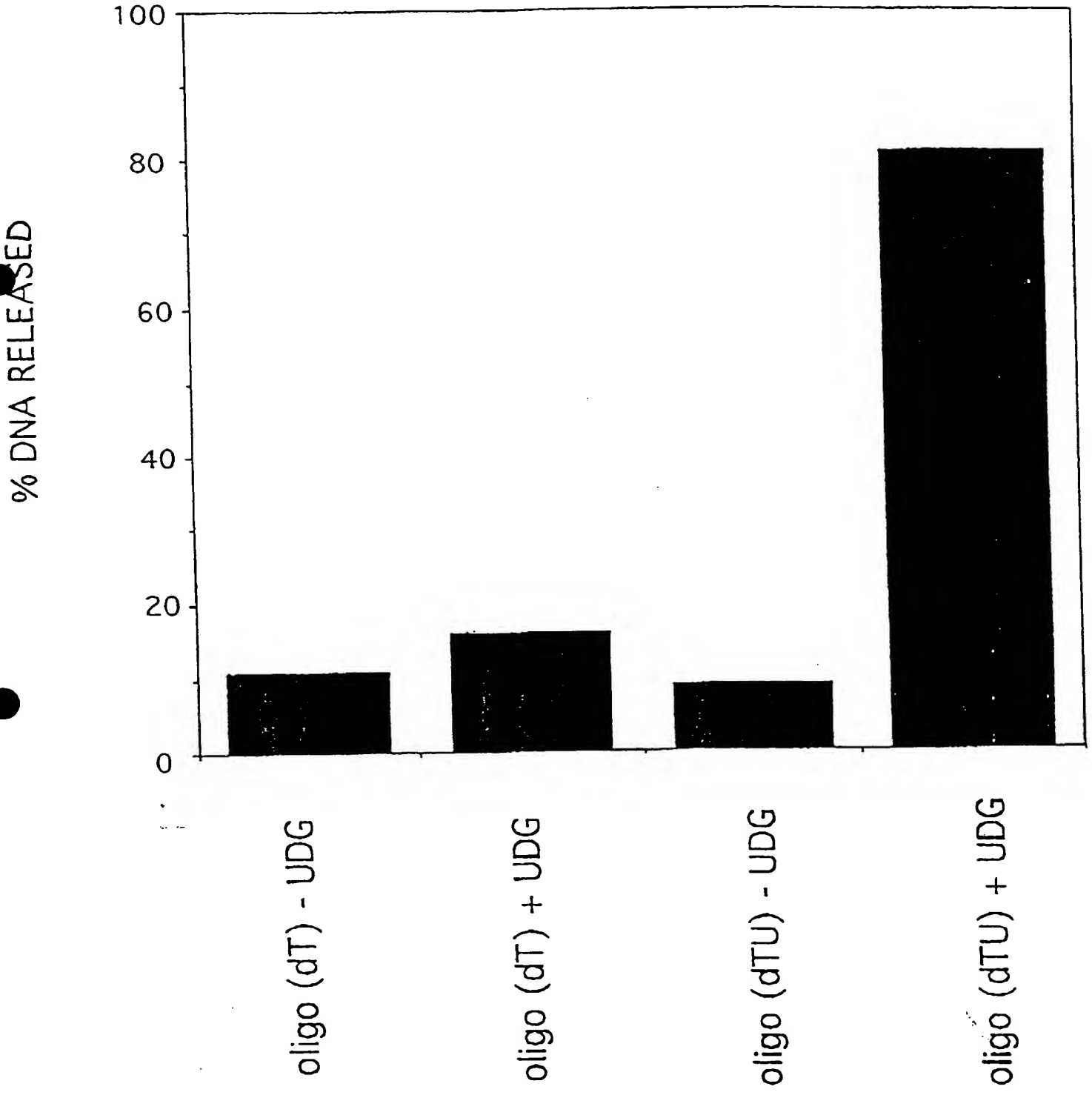
% DNA RELEASED
100
80
60
40
20
0
oligo (dT) - UDG
oligo (dT) + UDG
oligo (dTU) - UDG
oligo (dTU) + UDG

Figure 1

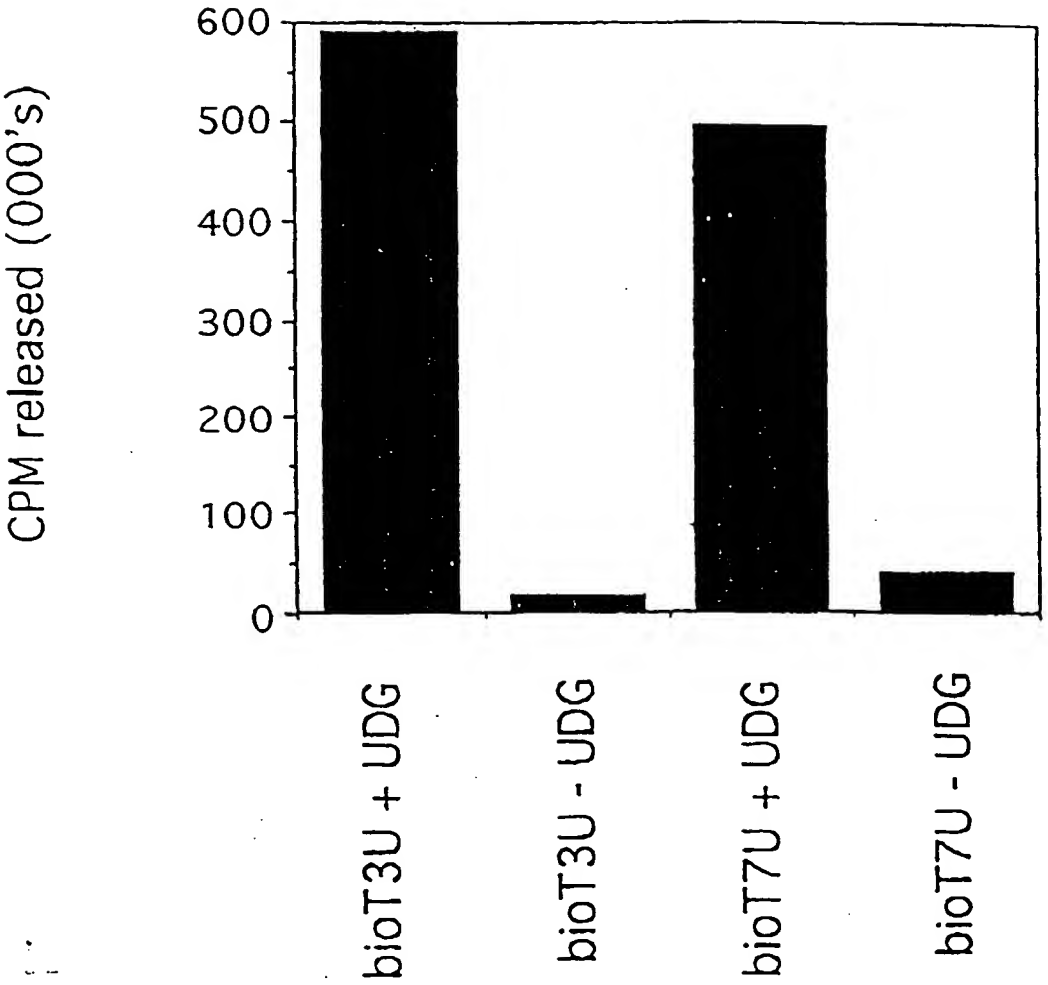
CPM released (000's)
600
500
400
300
200
100
0
bioT3U + UDG
bioT3U - UDG
bioT7U + UDG
bioT7U - UDG

Figure 2

[illegible]